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Foot-and-Mouth Disease Virus Exhibits an Altered Tropism in the Presence of Specific Immunoglobulins, Enabling Productive Infection and Killing of Dendritic Cells▽

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Foot-and-mouth disease virus (FMDV) causes an acute vesicular disease of farm animals. The development of successful control strategies is limited by an incomplete understanding of the immune response to FMDV. Dendritic cells (DC) mediate the induction of immunity to pathogens, but their role in FMDV infection of cattle is uncharacterized. Bovine monocyte-derived DC (moDC) were exposed to integrin-binding and cell culture-adapted strains of FMDV *in vitro*. MoDC were not largely susceptible to infection by integrin-binding FMDV but were susceptible to culture-adapted virus. Binding specific antibodies to integrin-binding FMDV at neutralizing or subneutralizing IgG concentrations significantly enhanced infection via CD32 (FcγR). Monocytes also expressed CD32 but were nonsusceptible to FMDV immune complex (IC) infection, indicating a requirement for additional factors involved in cellular susceptibility. Infection of moDC by the FMDV IC was productive and associated with high levels of cell death. Infected moDC were unable to efficiently stimulate FMDV-specific $CD4^+$ memory T cells, but exposing moDC to IC containing inactivated FMDV resulted in significantly increased T cell stimulation. Thus, neutralized FMDV concurrently loses its ability to infect susceptible cells while gaining the capacity to infect immune cells. This represents a change in the tropism of FMDV that could occur after the onset of the antibody response. We propose that IC could dynamically influence the anti-FMDV immune response and that this may explain why the early immune response to FMDV has evolved toward T cell independence *in vivo*. Moreover, we propose that DC targeting could prove useful in the development of effective vaccines against FMDV.

Foot-and-mouth disease virus (FMDV) causes a highly contagious acute vesicular disease affecting over 70 different animal species (1). While the disease itself is rarely fatal, it represents a significant barrier to trade and a threat to global food security. FMDV is the type species of the genus *Aphthovirus* within the family *Picornaviridae*. This family of viruses is characterized by their nonenveloped icosahedral particles, about 30 nm in size, which contain the single-stranded positive-sense RNA genome.

Field strains of FMDV use cell surface α_v integrins for attachment and internalization (25, 26, 28, 42). Under physiological conditions, the integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ are most likely to play a significant role in infection (26, 41), although following extended passage in tissue culture, some strains have dispensed with the requirement for integrins, instead utilizing the ubiquitously expressed heparan sulfate proteoglycan as their cellular receptor (27).

Following inhalation, the first rounds of viral replication take place in the epithelium of the soft palate and pharynx (1, 45), followed by the lung (3). Virus can then be detected in the serum from around 24 h postinfection, with the first evidence of FMDV at secondary replication sites in the epithelium of the feet and mouth seen at 2 to 3 days (3). The cytopathic nature of FMDV replication in epithelial cells causes local tissue destruction, leading to the formation of the vesicular lesions characterizing the disease, with associated inflammation and immune cell recruitment (1, 3).

The host response to FMDV is dominated by the production of high titers of virus-neutralizing antibody, detectable from 3 days postinfection (11, 29, 54). The production of early antibody is T cell independent in both mice (9) and cattle (29). Moreover, the T cell response to FMDV during infection of cattle is not pronounced, despite an absence of lymphopenia or generalized immunosuppression (29). However, there is evidence that T cells are required for immune protection following immunization against FMDV (19) and, unlike B cells, recognize antigens that are shared between FMDV serotypes, which is of interest for vaccine design (10).

Antibody produced during infection is important for FMDV neutralization, but antibody-bound virus (immune complexes [IC]) can also be preferentially taken up by cells of the immune system that express Fc receptors (FcR). These receptors specifically recognize the Fc regions of antibodies forming immune complexes. FcR exist in several forms and are expressed by natural killer cells, mast cells, B cells, polymorphonuclear cells, macrophages, and dendritic cells (DC) (reviewed in reference 48). In phagocytes, FcR-mediated uptake of antibody-neutralized pathogens results in degradation, but in antigen-presenting cells (APC), material endocytosed via FcR is preferentially used to stimulate the adaptive immune response with enhanced efficiency (14, 17, 34, 61).

DC are professional APC situated throughout the body,

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particularly underlying the skin and mucosal surfaces, where they act as sentinel cells, constantly sampling the external environment for signs of infection or tissue damage. They are derived from bone marrow precursors that differentiate in the periphery into immature DC. On sensing a threat to the host, DC undergo a complex multistep maturation program and migrate to the local lymph node (LN), where they present processed peptide antigens acquired at the infection site on both major histocompatibility complex (MHC) class I and II molecules. Mature DC also express high levels of T cell costimulatory molecules, which gives them the unique ability to stimulate a primary adaptive immune response. The study of DC interactions with pathogens is critical to understanding the ensuing immune response and therefore to the rational design of vaccines.

Little is known of the interactions of FMDV with bovine DC, and studies using porcine DC have drawn contradictory conclusions. Early studies (12, 20) reported a low-level infection of skin DC (20), whereas a more recent study (5) concluded that porcine skin DC were not susceptible to FMDV infection by either field strains or cell culture-adapted variants of the virus (5). Finally, porcine monocyte-derived DC (moDC) interacted with both integrin- and heparan-binding FMDV variants to restimulate memory B cell responses *in vitro* (23). There is also some evidence that the interaction of APC with FMDV immune complexes may be important during infection *in vivo*. Mason et al. showed that transfection of FcR into nonsusceptible cells allowed productive infection by antibody-complexed FMDV (37). Similarly, infection of porcine macrophages-monocytes occurred in the presence of antibody-complexed FMDV, but not with virus alone (6). Such interactions may have important functional consequences for immunity, for example, porcine natural-interferon (IFN)-producing cells require immune-complexed FMDV to induce type I IFN production *in vitro* (22).

In the current study, we investigated the interaction of FMDV with bovine DC. We demonstrate that bovine moDC are susceptible at low frequency to FMDV but become significantly more susceptible and are productively infected in the presence of FMDV bound by neutralizing antibody. We also show that infection of moDC by immune-complexed FMDV has functional consequences that could adversely affect the development of the specific immune response *in vivo*. Moreover, we identify a potentially useful novel FMDV vaccine strategy for the future.

MATERIALS AND METHODS

All reagents were purchased from Sigma (Dorset, United Kingdom) unless otherwise stated.

Animals. The cattle used were conventionally reared outbred British Holstein Friesians bred at the Institute for Animal Health (IAH), Compton, United Kingdom. Both males and females of a range of ages were included in the study.

Ethics statement. This study was carried out in strict accordance with the Animals (Scientific Procedures) act of 1986. The project license under which this work was carried out was reviewed and approved for submission to the Home Office by the ethics committee of the IAH on 22 November 2005. The Home Office, which administers the Animals (Scientific Procedures) act of 1986, approved and granted the license on 12 April 2006.

Cell culture. Cells were cultured at 37°C at 5% CO_2 in air. The fetal calf serum (FCS) used for cell culture was confirmed to be free from bovine viral diarrhea virus, which is a common contaminant capable of infecting DC and affecting their function.

(i) Isolation of bovine leukocyte subsets. Heparinized blood was diluted with phosphate-buffered saline (PBS) (Invitrogen, Paisley, United Kingdom) and centrifuged over Histopaque 1083. Peripheral blood mononuclear cells (PBMC) were collected from the interface and washed three times with cold PBS. For the isolation of monocytes, resuspended cell pellets were incubated with anti-human CD14 paramagnetic microbeads (Miltenyi Biotech, Surrey, United Kingdom) before collection from a Midimacs column (Miltenyi Biotech) according to the manufacturers' instructions. For $CD4^+$ T cell isolation, cell pellets were incubated with mouse anti-bovine CD4 (CC30; IAH, Compton), washed twice in PBS, and then incubated with rat anti-mouse IgG1 paramagnetic microbeads (Miltenyi) and collected as described above. The purity of the isolated cell populations was analyzed by flow cytometry and found to be at least 95%. Cell viability was assessed by Trypan blue staining and was at least 95%.

(ii) Differentiation of moDC from monocytes. Freshly isolated monocytes were adjusted to an appropriate concentration in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Autogen Bioclear, Wiltshire, United Kingdom), 5×10^{-5} M β-mercaptoethanol, 50 μg ml^{-1} gentamicin, 200 U ml^{-1} CHO-derived bovine recombinant interleukin 4 (IL-4), and 0.2 U ml^{-1} bovine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). The recombinant IL-4 was produced at the Moredun Research Institute by Sean Wattegedera and Gary Entrican in conjunction with the BBSRC/SEERAD Immunological Toolbox (grant numbers BBS/B/00255 and MRI/094/04). MoDC were differentiated over 6 days, with one-third of the medium replaced by fresh medium on day 3.

(iii) Cell lines. Baby hamster kidney (BHK-21) cells were maintained in Glasgow minimal essential medium (GMEM) supplemented with 10% FCS, 5% tryptose phosphate broth, 100 U ml $penicillin^{-1}$, 100 μg ml $streptomycin^{-1}$, and 2.5 μg ml amphotericin B^{-1}. Chinese hamster ovary (CHO-K1) cells were maintained in nutrient mixture F-12 Hams (HEPES modification) supplemented as described above.

FMDV stock. The integrin-binding O1K-Cad2 virus originated from a field isolate collected during a European outbreak in the 1960s, which was propagated initially in primary bovine thyroid cells at IAH, Pirbright. Working stocks were prepared by a single round of propagation in BHK-21 cells. Briefly, cell monolayers were washed once with PBS before the virus stock, diluted 1:6 in virus growth medium (GMEM supplemented with 1% adult bovine serum), was added. Virus was allowed to bind at 37°C for 30 min, after which further virus growth medium was added and the flasks were incubated until the cytopathic effect (CPE) was just evident. For maximum virus yield, the flasks underwent a freeze-thaw cycle at −70°C before clarification by centrifugation.

Sucrose gradient purification of FMDV. Purified virus was used for T cell proliferation assays to ensure that all detected proliferation was FMDV specific and to avoid nonspecific stimulating effects on moDC. Clarified virus was precipitated overnight using ammonium sulfate at a final concentration of 29% (wt/vol). The precipitated virus was pelleted by centrifugation and resuspended in phosphate buffer (PB) (40 mM PO_4, 100 mM NaCl, pH 7.6) with 1% NP-40. The virus was ultracentrifuged over a 30% sucrose-in-PB cushion to pellet it. The resuspended pellets were incubated with RNase (0.2 mg ml^{-1}) for 5 min before NP-40 was added to a concentration of 1%. The remaining cell debris was removed by centrifugation. The supernatant was loaded onto a 15 to 45% sucrose gradient and ultracentrifuged for 2.5 h. Fractions were drawn from the gradient, and optical density readings at 260 nm (OD_{260}) were used to identify virus-containing samples. The concentrations of the pooled samples were estimated using the following formula: (OD_{260} × total volume) = mg of virus. After a final ultracentrifugation step, purified virus was resuspended to 1 mg ml^{-1} in 40 mM HEPES, 100 mM NaCl, 0.75% ammonium sulfate, pH 7.5, and stored at −70°C. The purity of the virus preparation was confirmed by SDS-PAGE with Coomassie blue staining.

Titration of virus stocks. A 10-fold dilution series of the virus stock was prepared in PBS, and 100 μl per well of each dilution was added to PBS-washed BHK-21 cell monolayers in triplicate. Virus was bound for 15 min at 37°C before the addition of 3 ml per well of molten Eagle's overlay (0.6 g indubiose [Cipher-gen Biosystems Inc., Guildford, United Kingdom] dissolved in 25 ml of water added to 75 ml Eagle's overlay, 5 ml tryptose phosphate broth, 1 ml FCS, 100 U ml $penicillin^{-1}$, and 100 μg ml $streptomycin^{-1}$, all at 45°C). The plates were incubated for 24 h before visualization of plaques using methylene blue solution (0.1% [wt/vol] methylene blue and 10% formaldehyde in PBS). The titer of the virus was calculated based on the number of plaques formed in each well over the range of dilutions and is presented as PFU per ml. O1K-Cad2 was completely unable to form plaques on the CHO-K1 cell line, which lacks appropriate integrins but possesses heparan sulfate, and therefore, the virus cannot have contained any heparan sulfate-binding virions. The O1K-Cad2 stocks were therefore

considered genuinely integrin dependent, in contrast to O1-BFS, which gave similar titers on both BHK-21 and CHO-K1 cell lines.

UV inactivation of FMDV. UV inactivation was used to cross-link the FMDV RNA genome and prevent replication while retaining the capsid structure. The protocol used was based on that of Ostrowski et al. (44) with some minor modifications. FMDV was transferred to tissue culture plates on ice to a depth of no greater than 2 mm at a distance of 10 cm from the UV source (UVP, Upland, CA). The virus was exposed for 12 min to UV light at a wavelength of 256 nm with agitation every minute. To confirm complete inactivation, samples were subjected to serial passage on bovine thyroid cells.

Exposure of cells to FMDV *in vitro*. FMDV was added to cultures at a multiplicity of infection (MOI) of 10 and allowed to bind the cells in a small volume of serum-free medium for 30 to 60 min. Warmed complete medium was added after this time, and the cell cultures were incubated for 3 to 6 h for detection of viral nonstructural (NS) proteins or for 24 h for the detection of FMDV-induced CPE. This general protocol was modified for specific applications as indicated in the figure legends.

Flow cytometry. Following FMDV exposure, the virus inoculum was aspirated, and nonadherent moDC were collected by centrifugation. Loosely adherent cells were detached using warmed cell dissociation solution. The cells were fixed with 1% paraformaldehyde in PBS and permeabilized with 0.1% saponin in fluorescence-activated cell sorter (FACS) wash buffer (PBS with 1% bovine serum albumin, 0.1% sodium azide). To assay viral replication, cells were stained for FMDV nonstructural protein 3A using mouse monoclonal antibody 2C2 (13) or an isotype-matched control antibody and detected with fluorochrome-conjugated goat anti-mouse IgG2a (Southern Biotech, AL).

To detect dying cells in moDC cultures, 5×10^5 cells were pelleted by centrifugation and resuspended in annexin V binding buffer (MBL, Nagoya, Japan). The cells were incubated with 0.1 μg of annexin V conjugated to allophycocyanin (Invitrogen) and 0.5 μg of propidium iodide (PI) (MBL) for 10 min, protected from light. Before analysis, the cells were centrifuged to pellet them and resuspended in fresh binding buffer for flow cytometry.

Fluorescence data were collected using a Becton Dickinson FACScalibur with Cellquest software. Cells were gated for viability based on their forward scatter/side scatter (FSC/SSC) profile, with a minimum of 10,000 viable cells collected in each sample. The results were analyzed using FCS Express version 3 (De Novo Software).

CD32 detection and blocking. For microscopy, 5×10^5 moDC were cultured on uncoated glass coverslips. The antibody used to detect CD32 expression was CCG36 (IAH, Compton). In some experiments, CD32 on moDC was blocked by preincubation with CCG39 (IAH, Compton) at 1/10 dilution of culture fluid on ice. The cells were then washed with PBS before incubation with O1K-Cad2 IC for 1 h on ice. Unbound complexes were removed by washing the coverslips with PBS before fixation with 4% paraformaldehyde, and bound FMDV was then detected using the antibody IB11 (IAH, Compton), which recognizes a conformational epitope of the FMDV capsid. In every experiment, isotype-matched controls for primary antibodies were incorporated and did not alter the levels of infection compared to medium alone. Bound primary antibody was then detected using goat anti-mouse IgG1 Alexa 488-conjugated secondary antibody (Molecular Probes) at 1:500 dilution in PBS. To label nuclei for microscopy, 250 ng ml^{-1} solution of 4′,6-diamidino-2-phenylindole (DAPI) in PBS was added to the moDC. Coverslips were mounted onto Superfrost slides (BDH, United Kingdom) using Vectashield mounting medium (Vectalabs) and sealed with nail polish. Images were captured using an ×63 lens on a Leica SP2 confocal microscope equipped with 488 and 633 lasers. Images were minimally enhanced using Photoshop software (Adobe Systems), with equal brightness alterations being made to all images. For experiments to understand whether CD32 blocking also blocked viral replication, the above-mentioned protocol was applied with some adjustments; moDC were infected in tubes and, instead of fixation following IC binding and washing, were incubated at 37°C for 3 h to permit any bound FMDV IC to be internalized and to produce NS proteins. FMDV NS protein 3A was detected by flow cytometry as described above.

Immune complexes. Sera were collected from whole blood of two cattle both before and after immunization with the commercially available FMDV O1-Manisa vaccine. The sera were mixed with virus at an MOI of 10 and a dilution of 1/100. Some experiments were carried out using purified IgG at a dilution of 1/50 unless otherwise stated (IgG content roughly equivalent to the 1/100 dilution of crude serum). In the case of inactivated virus, a volume equal to the volume of live virus applied in parallel experiments was used. In all cases, immune complexes were formed at room temperature for 30 min before being added to the cells.

IgG was purified from serum collected from convalescent cattle. Two animals were needle challenged with O UKG 34/2001 and were used to infect a further two animals by direct contact. Blood was collected from these two naturally infected animals after viremia had passed and the presence of FMDV-specific IgG had been confirmed, at 17 days postinfection. Serum was also collected from one control naïve animal. IgG was purified using a HiTrap protein G HP column (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. The purity of the IgG samples was confirmed by gel electrophoresis.

Quantification of FMDV in infected cell supernatants. MoDC (1×10^6) were exposed to medium, O1K-Cad2, or O1K-Cad2 in immune complexes for 1 h on ice. As a positive control susceptible BHK cells were exposed to O1K-Cad2. Unbound virus was removed by washing the cells with PBS before they were resuspended in RPMI medium with 5% FCS and allowed to internalize the virus for 10 min at 37°C. The cells were pelleted by centrifugation and resuspended in citric acid buffer (0.1 M citric acid, pH 5.2, in 140 mM NaCl) to inactivate any viral particles left on the cell surface. After 5 min at room temperature, the cell suspensions were transferred to clean tubes to avoid any carryover of live virus before being diluted in complete medium (as described above for each cell type). Cell samples were split into 3 parts; time zero samples were placed on ice before the supernatants were removed. The remaining 2 samples were incubated for 3 or 5 h at 37°C. To ensure that no cell-associated virus contaminated the supernatants, the samples were centrifuged before being frozen at −80°C for later analysis. The FMDV titer in infected cell supernatants was determined by the standard World Reference Laboratory method used to calculate the 50% tissue culture infectious dose ($TCID_{50}$)/ml (31).

T cell proliferation assay. MoDC (1×10^3 per well) in proliferation medium (RPMI 1640 supplemented with 10% FCS, 5% nonessential amino acids [Invitrogen], 5 mM sodium pyruvate [VWR, Leicestershire, United Kingdom], 50 μg/ml gentamicin, and 0.5 μM 2-mercaptoethanol) were incubated with a range of antigens: a sample of the antigen used for vaccination (diluted 1/100 in proliferation medium), purified O1K-Cad2, or the same amount of purified O1K-Cad2 in IC formed with purified IgG from an FMDV-immune animal. In some cases, the virus was first UV inactivated. In each case, an MOI of 5 was employed, or an equivalent quantity in the case of UV-inactivated virus. It is not possible to concentration match purified FMDV with vaccine antigen; however, the vaccine antigen is a useful indicator of the animals' specific proliferative response to FMDV. Medium alone and pokeweed mitogen (2.5 μg/ml) were used as negative and positive controls, respectively. After 1 h of incubation of antigen with moDC, purified $CD4^+$ T cells from the same animals were added at 1×10^5 cells per well (an APC/responder ratio of 1:100). The cultures were incubated for 5 days before 37 Bq [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom) was added to each well. After overnight incubation, the plates were harvested onto filter mats, and incorporated radioactivity was assayed by liquid scintillation counting, using a 1450 Microbeta counter (Wallac, Finland).

RESULTS

Bovine moDC become highly susceptible to infection by FMDV in the presence of IgG from immune cattle. Initially, we investigated integrin expression on bovine moDC by flow cytometry using antibodies that are cross-reactive for bovine integrins. This showed that moDC express $\alpha_v\beta_3$ but not $\alpha_v\beta_6$, which is believed to be the main receptor for the virus *in vivo* (Fig. 1). MoDC were exposed to two isolates of FMDV (O1K-Cad2 and O1-BFS) that differ in their receptor usage; O1K-Cad2 is a field strain using α_v integrins for cell entry, whereas O1-BFS is a cell culture-adapted variant that has dispensed with its requirement for integrins and enters cells using the ubiquitously expressed heparan sulfate proteoglycan (27). Cells were exposed to the viruses at an MOI of 10 (i.e., a ratio of 10 infectious virus particles to one cell) for 6 h before detection of the viral NS protein 3A by flow cytometry as evidence of viral replication. MoDC cultures showed only a low level of infection by O1K-Cad2 (mean percentage of 3A-positive cells, 4.7), while in contrast, exposure to O1-BFS resulted in significantly higher levels of infection (mean, 25.1%; $P = 0.004$; Student's t test) (Fig. 2A). Confocal microscopy was used to confirm that our observations genuinely reflected FMDV replication in moDC. As expected, approximately 5%

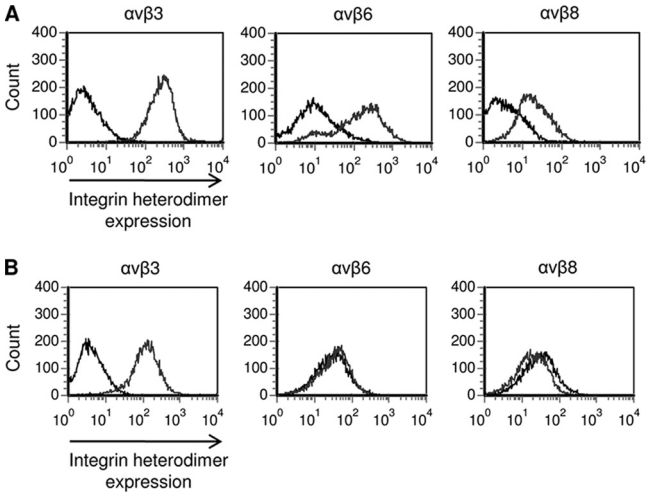

FIG. 1. Integrin expression on bovine MoDC. Shown is flow cytometry analysis of integrin heterodimer expression on positive-control cells (A) and moDC (B). The cells were labeled with antibodies recognizing bovine integrins (gray lines) or a concentration-matched isotype control antibody (black lines).

of moDC showed labeling for FMDV NS protein 3A (data not shown). Figure 2B shows the characteristic punctate distribution of FMDV 3A protein throughout the cytoplasm in infected moDC.

The above-mentioned experiments used detection of the viral 3A protein as a marker of virus replication. It is possible that such viral NS proteins are produced without full viral replication, so to understand whether moDC were supporting the production of new progeny virus, cells were exposed to O1K-Cad2 and O1K-Cad2 in IC. BHK cells were infected as a positive control. Both cell types were allowed to bind virus for 1 h on ice before they were washed and then transferred to 37°C for 10 min to permit internalization of bound virus. The outsides of the cells were then acid washed to inactivate any remaining input virus. At 0, 3, and 5 h postinfection, supernatants were collected from infected cultures and newly formed infectious virus was quantified. Figure 2C shows that an increase in the amount of infectious virus in cell supernatants from moDC was observed with time. As expected, the amount of virus produced was significantly greater following infection with O1K-Cad2 IC than with O1K-Cad2 alone ($P = 0.0026$ at 3 h and 0.0046 at 6 h; Student's t test).

As O1-BFS achieved a higher frequency of moDC infection than O1K-Cad2 (Fig. 2A), it seemed likely that the failure of O1K-Cad2 resulted from a lack of appropriate receptors for cell entry. Therefore, we asked whether immune-complexed O1K-Cad2 could bypass the requirement for integrin expression and instead use FcR to gain entry. Figure 2A shows that FMDV incubated with IgG from an immune animal was able to infect moDC at a significantly higher frequency than O1K-Cad2 alone (mean, 38.5%; $P = 0.004$; Student's t test). In contrast, IgG from the same animal before immunization had no effect, excluding a role for low-affinity natural antibodies. Similar experiments using heat-treated sera from immune animals produced the same results, thereby excluding a role for heat-labile complement components (not shown).

To understand whether the enhancement seen was dependent on a specific or subneutralizing concentration of antibody, checkerboard assays were carried out using the susceptible BHK cell line and moDC as targets, with CPE as the readout (Fig. 3). The concentration ranges of antibody with virus at an MOI of 10 that resulted in neutralization of FMDV infection in BHK cells overlapped substantially with the ranges that caused enhanced FMDV infection in moDC (1:8 to 1:64 and 1:4 to 1:16 using IgG from two different animals). Enhancement occurred over a wider range than neutralization, down to 1:128 and 1:256 IgG dilutions in two animals. The same pattern of observations was made using a range of MOIs of virus (not shown).

The above results show that bovine moDC are nonsusceptible to infection by a field strain of FMDV (most likely due to the lack of an appropriate integrin receptor) but are productively infected by antibody-complexed virus or cell culture-adapted FMDV that uses heparan sulfate as a receptor to enter cells. Antibody-mediated enhancement of FMDV infection occurred over a wide range of antibody concentrations, some of which also overlapped with IgG concentrations that were neutralizing on a susceptible cell line. We then went on to explore the implications of these findings.

CD32 is necessary but not sufficient for antibody-dependent enhancement of FMDV infection of bovine antigen-presenting cells. Figure 4 shows that FcγR (CD32) is uniformly and highly expressed on moDC (Fig. 4A, left). To confirm that antibody-dependent enhancement (ADE) of FMDV infection was mediated by CD32, blocking experiments were carried out using anti-CD32 antibodies. Figure 4B shows binding of immune-complexed FMDV to moDC (left) and shows that this binding is inhibited by anti-CD32 antibodies (right). As CD32 is highly expressed on the moDC surface, it was possible that anti-CD32 blocking antibody binding simply provided a steric hindrance to virus IC binding to receptors other than CD32. To exclude this possibility, cells were preincubated with either an anti-CD40 or an anti-CD1 antibody, which also recognize a high proportion of moDC. Antibody-bound moDC were exposed to FMDV IC for 1 h on ice, washed extensively, and then incubated for 3 h to allow infection to proceed (Fig. 4C). Infected cells were identified by detection of FMDV NS proteins by flow cytometry. Only CD32 antibodies caused a significant decrease in the percentage of infected cells relative to an isotype-matched control antibody ($P = 0.032$ for the difference between the level of infection with isotype control antibody versus that with anti-CD32 antibody; Student's t test), showing that ADE of FMDV infection in moDC is specifically mediated by IC binding to CD32.

CD32 is also expressed by other immune cell types, and so, to understand whether ADE of FMDV infection is a shared phenomenon, CD32 expression and the infection characteristics of another APC population were tested. Over 95% of the purified $CD14^+$ monocyte population that was used to differentiate the moDC was CD32 positive (Fig. 4A, right). Interestingly however, these cells were not susceptible to O1K-Cad2, including when the virus was in IC (Fig. 4D).

The above results show that monocytes are virtually nonsusceptible to antibody-enhanced FMDV infection but become highly susceptible after 6 days of culture with GM-CSF and IL-4. Figure 4E shows a time course experiment in which

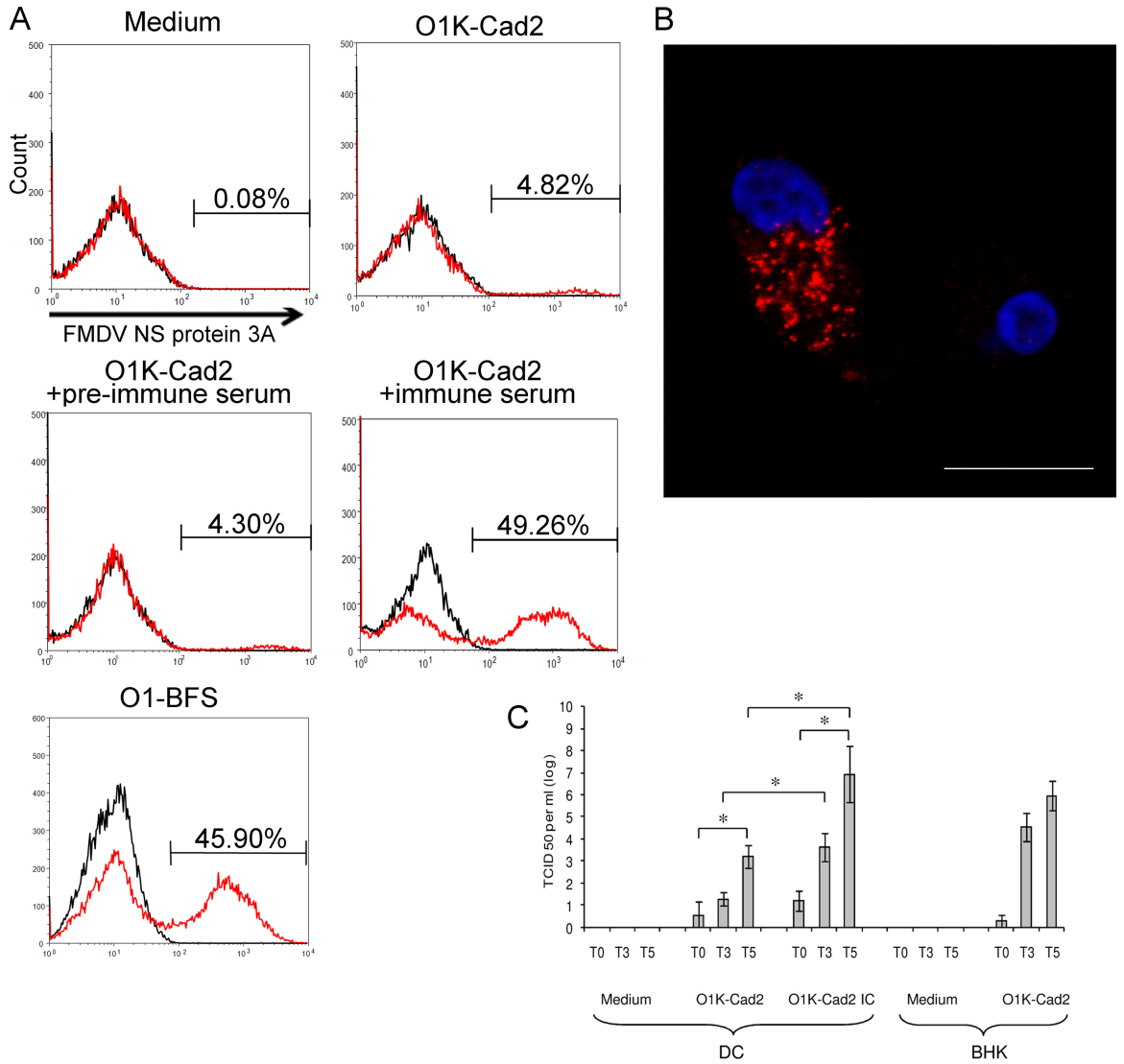

FIG. 2. MoDC become susceptible to FMDV only in the presence of IgG from immune cattle. (A) MoDC were exposed to FMDV isolates at an MOI of 10 for 6 h. Cells were incubated with medium, O1K-Cad2 alone, or IgG from cattle before or after vaccination against FMDV or, alternatively, with O1-BFS, which infects cells using heparan sulfate. FMDV NS protein 3A was detected by flow cytometry (red lines) and compared to labeling with an isotype control antibody (black lines). A representative experiment is shown. Significant enhancement of infection by O1K-Cad2 was seen in the presence of IgG from immune animals (mean percent infected, 3.6% compared to 38.5% with immune serum; $P = 0.004$; Student's t test; $n = 5$). (B) MoDC were cultured on glass coverslips and exposed to O1K-Cad2 at an MOI of 10 for 6 h. The cells were then labeled with antibodies recognizing FMDV NS protein 3A (red) and the nuclear stain DAPI (blue) before examination by confocal microscopy. The scale bar represents ~20 μm. (C) MoDC were exposed to medium, O1K-Cad2, or O1K-Cad2 IC. BHK cells were infected as a positive control. After inactivation of noninternalized virus, cell supernatants were collected at 0 (T0), 3 (T3), and 5 (T5) h postinfection. Live FMDV in supernatants was quantified. *, significant difference in mean FMDV titer at 0 and 5 h in moDC exposed to O1K-Cad2 ($P = 0.00007$) and O1K-Cad2 IC ($P = 0.004$) and between O1K-Cad2 and O1K-Cad2 IC at 3 h ($P = 0.0026$) and 5 h ($P = 0.0046$). The data presented are means ± standard deviations (SD) of 4 independent experiments; Student's t test was applied.

monocytes were tested for susceptibility to FMDV during their differentiation. Monocytes were isolated on day 0 and cultured toward moDC as detailed in Materials and Methods. Each day until day 6, a proportion of the cells were exposed to O1K-Cad2, O1K-Cad2 in IC, or medium as a negative control. After 6 h of exposure, the cells were analyzed for the presence of the FMDV 3A protein by flow cytometry (Fig. 4E). As early as the second day in culture, an increase in the susceptibility of the differentiating monocytes was seen. The frequency of cells positive for the 3A viral NS protein increased steadily during the 6-day period, reflecting a gradual acquisition of the infectible phenotype.

Taken together these results show that while CD32 is necessary for ADE of FMDV infection in moDC, it is not sufficient for infection of monocytes. Additional factors are required for cellular susceptibility to infection by FMDV IC and are acquired gradually during the monocyte-to-moDC transition over the course of 6 days.

FMDV replication correlates with increased moDC death and is associated with reduced specific T cell stimulation. The

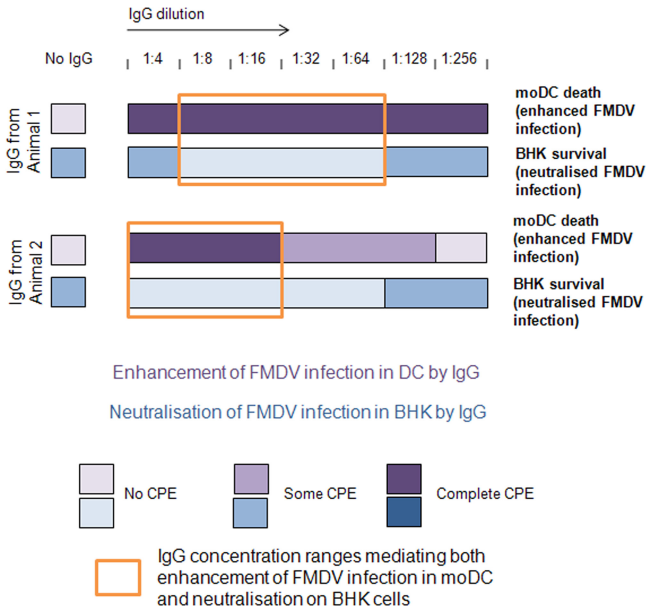

FIG. 3. The same antibody concentrations can act either to neutralize or to enhance FMDV infection. Purified IgG from either of 2 FMDV-immune animals was serially diluted before being mixed with O1K-Cad2 at an MOI of 10. Immune complexes protect BHK cells from infection by neutralizing FMDV, thus preventing integrin binding. In contrast, the same antibody permits FMDV to enter moDC via CD32. Cells were exposed to immune complexes formed under neutralizing and subneutralizing IgG concentrations and incubated for 24 h. CPE was detected under light microscopy and categorized as complete, incomplete, or absent.

above-mentioned results show that moDC support a productive FMDV infection. Figure 3A illustrates that moDC exposed to FMDV IC also showed signs of CPE characteristic of a lytic virus; however, some of the cells exhibited morphological signs of apoptosis, such as cell rounding and blebbing at the plasma membrane (Fig. 5A). To better understand these observations, cell death was studied in moDC exposed to O1KCad2, UV-inactivated O1KCad2, or cell culture medium. To quantify the level of cell death that was occurring and to investigate whether this represented a firm link between FMDV replication and moDC killing, infected moDC cultures were assessed by annexin V and PI staining. Annexin V binds phosphatidylserine, which is translocated from the inner to the outer plasma membrane during the early stages of apoptosis (59). PI is a DNA-intercalating dye that can gain entry only to cells with compromised membrane integrity, such as cells in the later stages of apoptosis or dying by necrosis (51). Initial experiments aimed to differentiate between apoptosis and necrosis in moDC cultures, but this was not possible, most likely due to the rapidity of FMDV-induced death or changes in membrane permeability that can occur during infection. Therefore, the combined percentage of cells staining with annexin V, PI, or both was used as a measure of total cell death. At hourly intervals during the 8-h infection period, samples of cells were removed and divided in 2. For each condition at each time point, the percentage of cells expressing FMDV NS proteins and the percentage of cells dying were assessed by flow cytometry (Fig. 5B). There was a close linear correlation between the percentage of moDC positive for NS protein and the percentage dying ($R = 0.929$; Pearson's correlation coefficient, where an R value of 1 is a perfect correlation). The difference between the absolute percentages of FMDV NS-expressing cells and dying cells is likely to be a result of the higher sensitivity of annexin V staining compared to the antibody used. In addition, it is expected that a background level of cell death will occur in these primary cell cultures. Repeating the experiment shown in Fig. 5B with heparan sulfate-binding FMDV reproduced the correlation, with an R value of 0.974 (not shown). In samples incubated with UV-inactivated FMDV, no NS proteins were detected and cell death was no higher than in medium-only cell populations (not shown). The absence of enhanced cell death in cultures incubated with inactivated virus further supports the notion of a causative relationship between FMDV replication and moDC death.

In vivo, high levels of infection of DC have the potential to impact the specific immune response. Therefore, we determined the effect of FMDV infection on the ability of moDC to stimulate FMDV-specific $CD4^+$ T cell proliferation using cells from FMDV-vaccinated animals. The $CD8^+$ T cell response in vaccinated animals is far weaker than the $CD4^+$ response and thus was not assayed in parallel. MoDC were incubated with either a sample of the vaccine antigen originally used to immunize the animals, purified O1K-Cad2, or purified O1K-Cad2 in IC. After 1 h of preincubation of moDC with the antigens, purified autologous $CD4^+$ T cells were added to the cultures and incubated for 5 days. Tritiated thymidine incorporation was assessed as a measure of T cell proliferation induced by infected moDC. Figure 5C shows that moDC were able to induce a high level of recall T cell proliferation in response to inactivated FMDV vaccine antigen. Similarly, moDC exposed to O1K-Cad2 induced good levels of T cell proliferation, probably the result of a lack of infection-related death coupled with presentation of the high levels of inactive FMDV known to exist in purified preparations. In contrast, moDC incubated with O1K-Cad2 IC induced significantly lower levels of proliferation, most likely as a result of CPE in the moDC. As FMDV does not infect bovine lymphocytes *in vitro* (data not shown) or *in vivo* (30), it is unlikely that the reduced $CD4^+$ T cell proliferative response was the result of a direct interaction with the virus.

In summary, there is a strong linear correlation between FMDV infection and moDC death. The requirement for live virus to induce death in moDC cultures implies a causal relationship, which is also associated with a significantly decreased ability of moDC to stimulate memory responses to FMDV *in vitro*.

The increased interaction of FMDV IC with DC can be used to target inactivated FMDV to the cells for enhanced T cell stimulation. A T cell proliferation assay was also used to establish whether incubating moDC with immune-complexed, UV-inactivated FMDV could overcome the adverse effects on T cell proliferation that were observed with live FMDV IC. In these experiments, moDC and $CD4^+$ T cells from vaccinated animals were incubated with medium alone, a sample of the vaccine originally used to immunize the animals, purified UV-inactivated O1K-Cad2, or purified UV-inactivated O1K-Cad2 in IC (UV-IC). UV-IC stimulated significantly more T cell proliferation than UV-inactivated O1K-Cad2 (Fig. 6). Com-

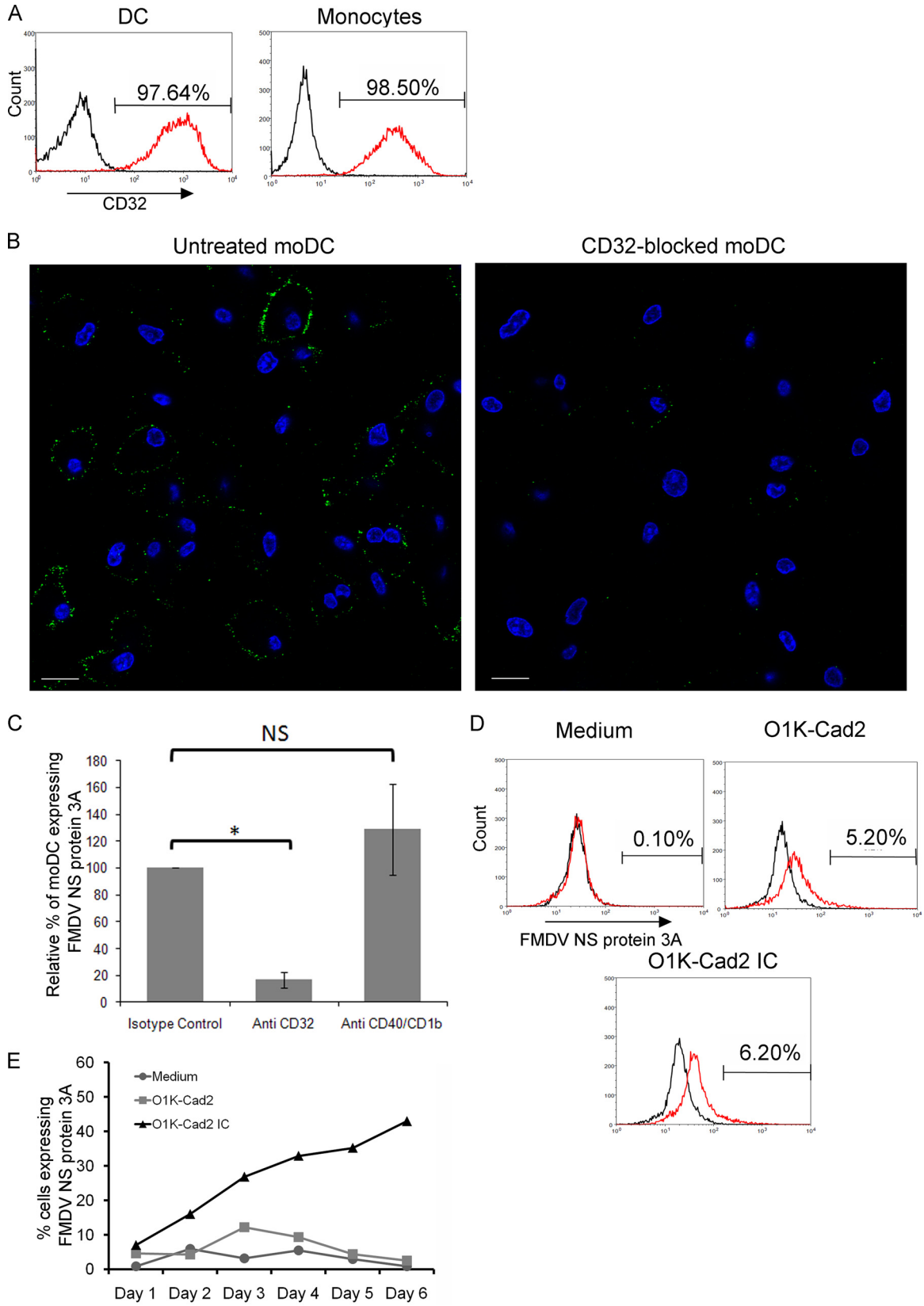

FIG. 4. CD32 expression is necessary but not sufficient to mediate antibody-dependent enhancement of FMDV infection. (A) Bovine moDC (left) or monocytes (right) were labeled with antibodies to CD32 (red lines) or an isotype control antibody (black lines). The mean percentage of moDC expressing CD32 was 96.5 (SD, 3.5; $n = 3$), and that of monocytes was 99.1% (SD, 0.46; $n = 3$). (B) O1K-Cad2 IC at an MOI of 10 were incubated with moDC on ice for 1 h before detection of FMDV capsids (green) and nuclei (blue) (left). A CD32-blocking antibody (CCG39) was

pared to UV-inactivated virus alone, an increase in counts per minute (cpm) of up to 15-fold was observed using IC. Thus, by exploiting the CD32-mediated targeting of moDC, inactivated FMDV can be delivered to the cells in a way that enables increased stimulation of specific T cell responses.

DISCUSSION

We used bovine monocyte-derived DC to strengthen our understanding of the interaction of FMDV with the host immune system. There is convincing evidence that moDC have a critical role to play *in vivo* in immunity to acute infections (35, 46, 47). Based on these studies, our working model was that cytopathic FMDV infection of epithelial cells induces inflammation in the skin, resulting in recruitment of monocytes to the tissue site, where they differentiate into monocyte-derived macrophages and DC. These cells would then be exposed to high titers of live virus from vesicle fluid before leaving the tissue site to stimulate the adaptive immune response in the regional LN. Our studies were focused on understanding the nature and consequences of the interaction between FMDV and DC in driving the subsequent immune response.

Elements of this proposed model were investigated using *in vitro* studies. MoDC were first exposed to integrin-binding FMDV at a high MOI, calculated to approximate the concentration of live virus in vesicle fluid. After 6 h, around 5% of cells contained FMDV NS protein, in contrast to approximately 50% of BHK cells, which are considered highly susceptible to the virus, infected in parallel (not shown). This level of moDC infection was not due to a heparan sulfate-binding viral variant, as the virus preparations were free from such contamination (see Materials and Methods), but might have been due to a subpopulation of FMDV able to utilize $\alpha_v\beta_3$ integrins, which are expressed by moDC, or to nonspecific uptake of the virus. These data implied that an entry block was the main barrier to FMDV infection of moDC, which was supported by the finding that the integrin-independent heparan sulfate-binding FMDV isolate O1-BFS was indeed able to infect the majority of moDC. Our results are consistent with studies in immature porcine moDC, which are also productively infected *in vitro* with a tissue culture-adapted isolate of FMDV (15).

These results are consistent with work on DC and other picornaviruses. MoDC express the poliovirus receptor and are susceptible to infection (60). Similarly, human moDC could be infected by echovirus, but not by coxsackie B virus, and this again was related to appropriate receptor expression (33). FMDV requires only that it be internalized by the cell into a mildly acidic compartment where it can uncoat its genome and begin replication. While there is some evidence that poliovirus was also able to utilize FcR to infect cells, this seems applicable only to cell lines exposed at an MOI of 100,000, which even then results in inefficient infection in the absence of the poliovirus receptor (2). Thus, it is generally accepted that poliovirus infection relies on conformational changes to the capsid following specific receptor binding in order for the virus to uncoat (38). It is not surprising, then, that FMDV, but not poliovirus, can take advantage of a number of routes of uptake.

It has been previously documented that FMDV IC associated with FcR-bearing cells more readily than virus alone (6, 39, 52), but the interaction of FMDV IC with conventional (nonplasmacytoid) DC had not been explored. The extent of moDC infection in the presence of FMDV IC was significantly greater than that in cells exposed to virus alone. This enhancement of infection was mediated solely by IgG binding to FMDV and did not involve complement components or natural antibodies. Blocking of CD32 on moDC inhibited IC binding to the cell surface and so inhibited infection. Enhancement occurred in the absence of the specific receptor for FMDV and across a wide range of virus-antibody concentrations, including neutralizing conditions.

These assays were carried out to permit the consideration of ADE of FMDV infection in the context of current knowledge of other viral systems. The possible mechanisms of increased levels of viral infection in the presence of antibody can be summarized as follows; (i) enhanced direct endocytosis of the virus stimulated by FcR signaling following IC binding; (ii) increased adhesion of virus IC to the surfaces of FcR-bearing cells, which allows better access/easier binding of the virus to its natural receptor; (iii) increased adhesion of virus IC to the surfaces of complement receptor-bearing cells, allowing better access for the virus to its natural receptor; and (iv) facilitation of membrane fusion of enveloped viruses by antibody or complement components bound to the virus IC (reviewed in reference 57). Most commonly, ADE occurs in the presence of the specific viral receptor, for example, with dengue virus, HIV, and hepatitis A virus, another picornavirus (7, 16, 36). In these systems, the antibody that enhances these infections is nonneutralizing, as macrophages or DC are normally susceptible to these viruses, albeit at a lower level.

FMDV infection through IC is distinct from that of many other viruses. The current study has shown that moDC are not normally widely susceptible to FMDV infection, as they lack

applied before immune complexes, resulting in reduced FMDV binding to moDC (right). The scale bars represent ~20 μm. (C) moDC were preincubated on ice with a CD32-blocking antibody (CCG39), an isotype control antibody, or an irrelevant but binding antibody recognizing either CD40 or CD1b. O1K-cad2 IC at an MOI of 10 were allowed to bind on ice before being washed. After 3 h at 37°C, FMDV NS protein 3A was detected by flow cytometry. The percentage of infected moDC in isotype control-exposed wells was taken as the baseline, and relative percentages under the other conditions were calculated. A significant decrease in the mean percentage of infection by O1K-Cad2 IC was seen following CD32 blocking ($P = 0.032$; paired Student's t test; $n = 3$), but not after incubation of cells with an irrelevant antibody ($P = 0.2$). *, $P = 0.032$ by paired Student's t test; NS, not significant. (D) A day after isolation, monocytes were exposed (MOI, 10) to O1K-Cad2, O1K-Cad2 IC, or medium. After 6 h, the cells were labeled with antibodies recognizing FMDV NS protein 3A (red lines) or with an isotype-matched control antibody (black lines). The inclusion of antibody with FMDV did not affect infection levels ($P = 0.86$; Student's t test; $n = 4$). (E) Monocytes were isolated and cultured in moDC medium. Each day for 6 days, a sample of the cells was exposed to medium, O1K-Cad2, or O1K-Cad2 IC at an MOI of 10 for 6 h. FMDV NS protein 3A was detected by flow cytometry. A representative experiment out of two is presented; days 1 to 3 were repeated a further 3 times, showing the same result.

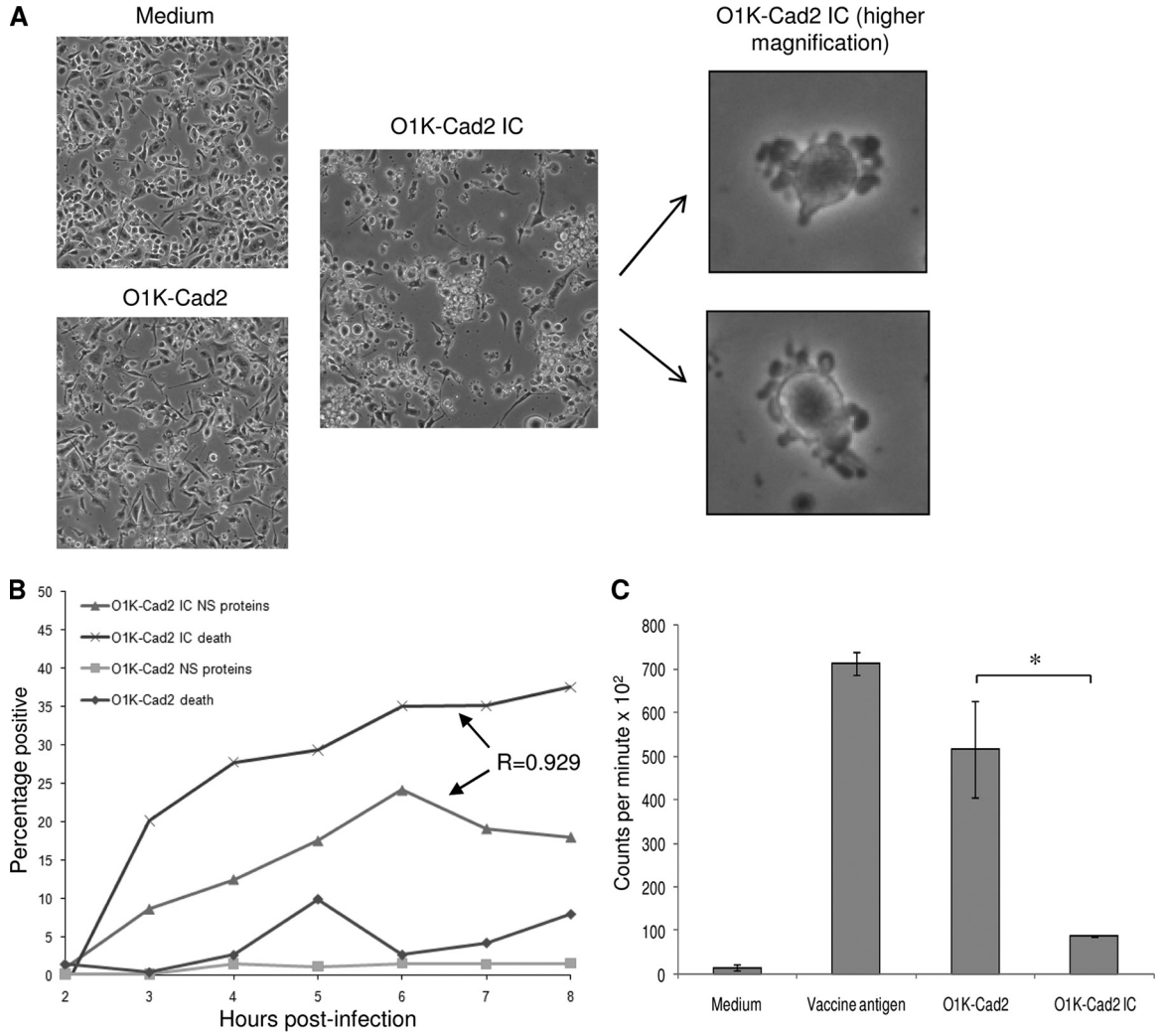

FIG. 5. FMDV infection of moDC is closely associated with cell death and reduced ability to stimulate FMDV-specific T cell proliferation. (A) MoDC were exposed to medium, O1K-Cad2, or O1K-Cad2 IC at an MOI of 10 for 6 h. Phase-contrast microscopy captured the high levels of dying cells, including some exhibiting classical apoptotic morphology (high power; right). (B) MoDC were exposed to medium, O1K-Cad2, or O1K-Cad2 IC at an MOI of 10 for 1 to 8 h. Samples of each culture were labeled with antibodies against FMDV NS protein 3A or with annexin V conjugated to APC, and PI. Death was defined as staining with annexin V, PI, or both. The data presented are relative to medium-only values; $R = 0.929$ (Pearson's correlation coefficient) for the linear relationship between the proportion of cells positive for FMDV NS proteins and the proportion dying. (C) Purified $CD4^+$ T cells from an FMDV-vaccinated animal were added to autologous moDC at a ratio of 1 DC to 100 T cells, with either medium, a sample of the antigen used for vaccination, purified O1K-Cad2, or purified O1K-Cad2 in IC at an MOI of 5. After 5 days, [3H]thymidine was added to each well and incubated overnight before harvesting and liquid scintillation counting of the cells for incorporated radioactivity. Pokeweed mitogen (PWM) was used as a positive control, and in all cases, counts exceeded 350,000 cpm (not shown). A representative data set is presented as cpm ± SD. *, $P = 0.02$; Student's t test. All 3 replicates using cells from 2 different animals were significant at this level or above.

the receptor required by the virus. Infection by FMDV IC does not involve complement components and is blocked by anti-CD32 antibodies, conclusively demonstrating for the first time in a natural target species that CD32 can mediate infection of immune cells, as previously hinted at by the work of Mason et al. and Guzylack-Piriou et al. (22, 37). Importantly, FMDV IC infection of moDC occurred under conditions that were neutralizing on susceptible cells. Effectively, FMDV IC infection of moDC represents a situation of altered tropism of the virus; unlike dengue virus or HIV, which normally infect APC and whose infection is genuinely "enhanced" in the presence of antibody. FMDV can concurrently lose its ability to bind and infect its normal target cells and gain an ability to productively infect moDC.

As FMDV is a lytic virus in susceptible cells but undergoes abortive replication following IC-mediated infection of porcine natural interferon-producing cells (22), we next investigated the functional effects of moDC infection by the virus. FMDV replication was productive and correlated closely with moDC death. While a causative link between virus replication and cell death remains to be conclusively shown and we were unable to define the exact mechanism of death, the lack of moDC killing in the presence of the same amount of UV-inactivated FMDV does support this conclusion. Furthermore, under conditions

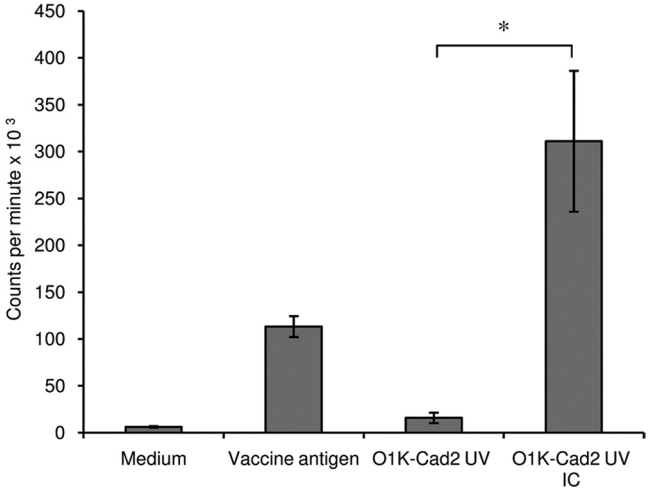

FIG. 6. Using IC to target inactivated FMDV to moDC results in significantly increased T cell stimulation. Purified $CD4^+$ T cells from an FMDV-vaccinated animal were added to moDC with either medium, a sample of the antigen used for vaccination, purified UV-inactivated O1K-Cad2, or purified UV-inactivated O1K-Cad2 in IC at an MOI of 5. After 5 days, [3H]thymidine was added to each well and incubated overnight before harvesting and liquid scintillation counting of the cells for incorporated radioactivity. PWM was used as a positive control, and in all cases, counts exceeded 350,000 cpm (not shown). A representative data set is presented as cpm ± SD. *, $P = 0.05$; Student's *t* test. Two further independent experiments gave *P* values in the range 0.005 to 0.078.

where a high percentage of moDC were infected, specific T cell responses to FMDV were significantly reduced. This combination of moDC killing and consequent lack of T cell stimulation could plausibly have significant consequences for the *in vivo* immune response during FMDV infection.

A CD32-mediated alteration in lytic virus tropism might be predicted to adversely affect all CD32-expressing cells. Bovine monocytes are CD32 positive and so were exposed to FMDV IC but were nonsusceptible to FMDV infection. Such differences in susceptibility between monocytes and DC have been reported for a range of viruses, including herpes simplex virus (HSV), echovirus, and poliovirus, where monocytes were found to be more resistant to infection than DC (33, 40, 60). In contrast, monocytes are more susceptible than moDC to CPE following influenza virus infection (8). However, our results seem to contradict well-established evidence of monocytes as targets of ADE of viral infection. The best-characterized example of this is dengue virus. Work by Kou et al. showed that while approximately 5% of human monocytes were permissive to dengue virus infection at an MOI of 5, this was increased to around 10% in the presence of highly diluted anti-dengue virus antibodies (32). Blocking CD32 or CD64 reduced ADE by about 50 to 70% (32), indicating that both these FcR may play a role in support of the work of Littaua et al. (36) but not excluding the involvement of other receptors. Further complicating the issue, monocyte infection by uncomplexed dengue virus has been reported in the range of 30 to 60% (18, 58), making interpretation of the data concerning ADE challenging. Encouragingly, the finding that monocytes are nonsusceptible to FMDV IC infection is consistent with *in vivo* data, where no FMDV replication is detected in PBMC of infected animals (62).

Lastly, we asked whether the potentially harmful effects of FMDV IC targeting of moDC could be conceptually reversed as an antigen-targeting strategy to induce effective T cell stimulation. IC of UV-inactivated O1K-Cad2 were able to interact with moDC to induce significantly more T cell proliferation than uncomplexed inactivated virus. As moDC do not express the receptor for O1K-Cad2 cell entry, uncomplexed virus enters moDC via nonspecific mechanisms, perhaps by macropinocytosis. By enabling virus to access the cells through the FcR, it was possible that the increased proliferation induced was due to a simple increase in the amount of antigen in the moDC. However, FcR-mediated uptake not only affects the amount of antigen taken up, but the characteristics of the endocytic route itself allow increased levels of antigen presentation on both MHC class I and II, coupled with efficient DC maturation, which is required for T cell stimulation (4, 49, 53, 55). An intriguing prospect that we plan to explore is the possibility of enhancing both CD4 and CD8 T cell responses in cattle *in vivo* using DC-targeted antigens as a vaccine. CD8 T cell responses are able to mediate protection *in vivo* from a range of intracellular pathogens, including respiratory syncytial virus (56) and malaria parasites (50), while $CD4^+$ T cell responses to FMDV vaccination correlate with protection against challenge (19, 24). The frequency of FMDV-specific CD8 T cells is very low post-FMDV vaccination (21), and our hypothesis is that targeting antigen to DC in a manner that may increase MHC class I presentation during the initiation phase of the immune response will result in enhanced CD8 T cell responses and improved protection.

In summary, the data presented here allow us to make new connections in our knowledge of the immune response to FMDV and to begin to explain some of the observations of previous studies. Our working model is that monocytes in blood are not infected either by virus alone or by FMDV IC later in infection. This agrees with the lack of replication of FMDV in PBMC (62) even in the face of high titers of live virus in blood (43). As FMDV replication occurs in the skin epithelium, monocytes will be recruited as a result of inflammation and begin to differentiate into monocyte-derived macrophages and moDC. As this occurs, the developing monocytes will become susceptible to FMDV IC infection and subsequent death, as will moDC that were present in the tissue site before the onset of significant antibody production. This would effectively deplete the local population of APC that were destined to stimulate the FMDV-specific adaptive immune response while leaving the animal able to respond to unrelated antigens, as demonstrated in cattle *in vivo* (M. Windsor, unpublished data). This is also consistent with the lack of pronounced T cell response that is consistently observed during FMDV infection of cattle (29). Extensive moDC killing by FMDV IC was observed in as little as 6 h postinfection, making it highly unlikely that these infected cells would be able to migrate to the LN and process and present FMDV antigens to T cells. This is supported by the finding that $CD8^+$ T cell responses to FMDV were in fact greater following vaccination than following infection (21). In poliovirus infection, in contrast, moDC death occurred at around 24 h postinfection, which allowed adequate time for T cell stimulation (60). Despite local APC death,

FMDV IC would be unable to infect epithelial cells, which would limit production of new virus and enable eventual control of the infection.

In conclusion, the current study has begun to illuminate the characteristics of the interaction of FMDV with bovine DC. These results have allowed a model to be proposed that predicts outcomes that can be easily reconciled with the available data concerning immune responses to FMDV in cattle. Despite the importance of antibody in the control of FMDV infection, this study has illustrated the potential importance of FMDV IC in compromising elements of the immune response, which has largely been neglected to this point. The study has identified the potential of rational vaccine designs to efficiently stimulate the immune system by targeting inactivated FMDV antigen to DC. Further work is planned to define the additional factors controlling susceptibility to CD32-mediated FMDV IC infection and to explore the potential of DC-targeted vaccines to successfully stimulate a multifaceted immune response to control FMDV.

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